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Award Number: DAMD17-00-1-0336

TITLE: The Functional Significance of the CSVTCG-Specific Receptor in Breast Carcinoma Progression

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REPORT DATE: June 2001

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	June 2001	Annual Summary (1 Jun 00 - 31 May 01)

4. TITLE AND SUBTITLE
The Functional Significance of the CSVTCG-Specific in Breast Carcinoma Progression

5. FUNDING NUMBERS
DAMD17-00-1-0336

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

We have isolated a novel CSVTCG-specific tumor cell adhesion receptor and shown by immunohistochemical staining of human breast tumors that the receptor localizes to malignant ductal epithelium while no staining of epithelium in normal and benign tissues was observed. Preliminary studies suggest that the CSVTCG-specific receptor may function to promote the invasive behavior of breast epithelium and contribute to the development of malignancy. To test this hypothesis, we propose to transfect full-length receptor cDNA in the sense or antisense orientation in order to either over-express or block receptor expression in breast carcinoma cell lines. We will use vector constructs that constitutively express the green fluorescent protein in order to enable localization of tumor cells in our animal studies. Stably transfected cell lines will then be evaluated for their in vitro cell adhesive and invasive activities as well as their capacity to metastasize and form tumors in athymic mice. These studies should provide information on the role of the CSVTCG-specific receptor in breast cancer progression and identify a new target for the development of anti-tumor therapeutics.

14. SUBJECT TERMS
Thrombospondin-1, metastasis, invasion, tumor marker

15. NUMBER OF PAGES
8

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Research Report

Thrombospondin-1 (TSP-1) is a high molecular weight glycoprotein composed of three identical disulfide-linked polypeptide chains. Each chain is composed of different domains and one of them the type I repeat domain has the cysteine-serine-valine-threonine-cysteine-glycine (CSVTCG) repeating sequence. Our laboratory has shown that the CSVTCG sequence of TSP-1 functions as a tumor cell adhesion domain and CSVTCG peptides as well as anti-CSVTCG antibodies have anti-metastatic activity in a murine model of lung metastasis. We have also isolated a novel CSVTCG-specific TSP-1 receptor and have shown that anti-receptor antibodies blocked breast cancer adhesion and invasion as well as human breast cancer progression in athymic mice.

These studies suggest that the CSVTCG-specific receptor functions in the promotion of the invasive behavior of breast cells and contributes to the development of malignancy. To test this hypothesis, MDA-MB-231 breast cancer cells were transfected with the full-length receptor cDNA in the sense and the antisense orientation in order to over-express and block the receptor expression. The pTracer-SV40 vector (Invitrogen,CA) was the vector originally proposed in the grant to be ligated to the gene of interest. The vector contains a gene for a green fluorescent protein (GFP) that has its own CMV promoter. Transfection was done by using Superfect reagent (Qiagen,CA) and only one week after selection with zeocin, cells expressing GFP were no longer found. With no marker protein in my cells I had to look for another vector that would stably express GFP. I changed to pTracer-CMV2 (Invitrogen,CA) and ligated it to the sense and the anti-sense receptor cDNAs. According to the company, the EF-1α promoter that drives the GFP expression can give high level constitutive expression of GFP. Again, I found that cells expressing the GFP protein were unstable and decided to change the transfection system. I chose the retroviral vectors and by the production of retroviral particles I infected the target cells.

The first retroviral vector was the pLXSN (Clontech,CA). The pIRES2-eGFP was used to subclone the internal-ribosome-entry-site (IRES) and the eGFP into the pLXSN. The IRES permits the translation of two open reading frames from one messenger RNA. The construction of this vector was not successful because of mutated restriction sites in the vector. Dr. Warren S. Pear (Univ. of Pennsylvania) kindly offered the retroviral

vector MigRI that contains the IRES site and the GFP. After cloning of the sense and antisense receptor cDNA in the MigRI, successful retroviral infection of MDA-MB-231 cells was achieved. The cells are sorted for their brightest green fluorescent color using a flow cytometer and then clones are generated by a dilution plating technique.

Preliminary data have shown that the cells transfected with receptor cDNA in the sense direction are more adherent to TSP-1 compared to the vector control transfected cells (figure 1). In 96 well plate, duplicate wells are covered with TSP-1 (40μg/ml), fibronectin (40μg/ml) and 1% BSA. The wells are dried out overnight and then blocked with BSA. 100μl of a suspension containing 25,000 cells are plated in the protein covered wells and incubated at 37°C for 1 hour. The non-adherent cells are removed and the wells are washed with a Hepes buffer. The total cell associated protein is determined by dissolving the attached cells directly in the wells with 150 μl of the BCA solution (Pierce Chemical Co.) The plate is incubated at 60°C for 20 minutes and the absorbance of each well is measured at 562 nm with a microtiter plate reader (Biotek, VT). Cells adhering to BSA are considered background while cells adhering to fibronectin are the positive control.

In addition, northern blot analysis revealed that the receptor transfected cells expressed higher levels of receptor mRNA (figure2). Total RNA is isolated from the cells by Rneasy Total RNA kit (Qiagen, CA) following the manufacturer's directions. 10 μg of total RNA is electrophoresed on 1% agarose/formaldehyde gel and blotted onto nylon membrane. The membrane is hybridized with an HRP-labeled receptor cDNA probe generated using the North2South kit (Pierce,IL). A chemiluminescent solution is used for development and then the blot is exposed to film.

The significance of these findings with respect to protein expression is currently being investigated. The stably transfected clones will then be further evaluated for their in vitro cell adhesive and invasive activities as well as their capacity to metastasize and form tumors in athymic mice. This retroviral vector will also be used for infection of other breast cell lines and their in vitro as well as their in vivo functions in cancer progression will be investigated.

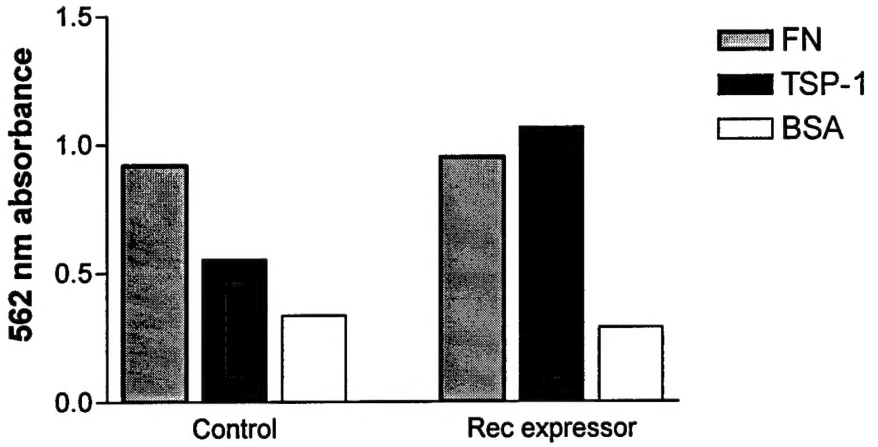

Fig. 1. Adhesion assay of vector control transfected and CSVTCG-specific receptor transfected MDA-MB-231 cells.

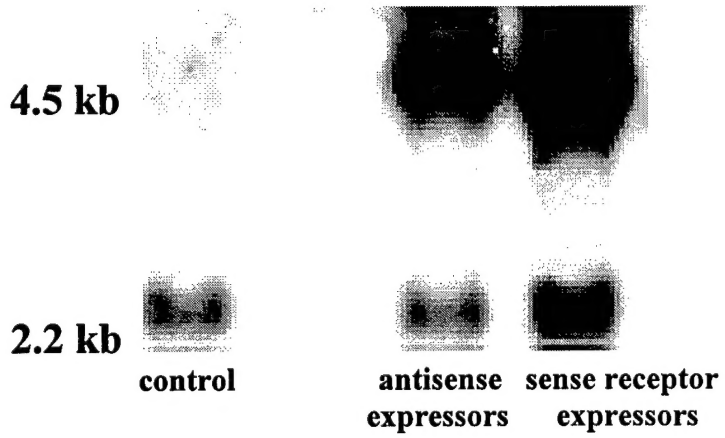

Fig. 2. Northern blot analysis of a mixed population of vector control cells, Antisense receptor expressing and sense receptor expressing cells.

Key Accomplishments

- Construction of pTracer-SV40 vector + CSVTCG specific receptor cDNA
- Transfection of MDA-MB-231 cells with the above DNA using the Superfect reagent
- Loss of fluorescence of the cells and change of vector
- Evaluate appropriate vector for cell transfection
    - Construction of pTracer-CMV2 vector + CSVTCG specific receptor cDNA
    - Transfection of MDA-MB-231 cells with the above DNA using the Superfect reagent
    - Loss of fluorescence of the cells
    - Change of the *in vitro* transfection conditions
    - Change of vector again! The pLXSN, pIRES2-eGFP and the CSVTCG specific receptor cDNA are used to construct a retroviral vector with eGFP, IRES and the desired insert.
    - Use a vector control to infect MDA-MB-231 cells. Successful infection with fluorescence that is detectable for a long period of time
    - Construction of the vector failed because of mutated restriction sites
    - Change of vector again! MigRI is the retroviral vector used.
    - Construction of MigRI with the CSVTCG specific receptor cDNA and the antisense cDNA
- Infection of MDA-MB-231 cells with the sense and antisense receptor cDNA
- Generate clones of vector control, sense and antisense receptor expressing cells by a dilution plating technique

Reportable Outcomes

In this first year of research no reportable outcomes have resulted from this award.